Design, Development, and Characterization of Imiquimod-loaded Chitosan Films

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Abstract

Basal Cell Carcinoma (BCC) is a common malignant tumor in Caucasians and accounts for 95% of non-melanoma skin cancers in the United States. BCC is primarily caused by UV radiation and is slow growing, although if left untreated, has high potential for spread to surrounding tissues. Non-surgical treatment of superficial BCC involves the use of Aldara™ cream containing 5% w/w imiquimod as the active ingredient. Imiquimod (Aldara™), a Toll-like receptor (TLR) agonist, is an immune response modifier with potent indirect antiviral activity. The topical delivery of imiquimod is desired over oral administration to eliminate the first-pass metabolism of drug in the liver while maximizing the drug concentration at the affected area. However, cream dosage form suffers from the disadvantages such as dose variability, poor drug availability due to incomplete release of the drug and poor patient compliance. We hypothesized that the sustained release of a therapeutic dose of imiquimod from the film would result in an effective treatment, while eliminating the need for daily cream application and the dosing variations associated with it. In order to test this hypothesis, imiquimod containing chitosan films were manufactured to achieve the desired controlled delivery of imiquimod and characterized for drug physical form, drug loading, content uniformity, and release behavior. In addition, effect of varying concentrations of Imiquimod and molecular weight of chitosan was studied on the release characteristics of the film. In-house HPLC method was developed to analyze the drug concentrations during the release studies. The X-ray diffraction (XRD) data and content uniformity studies demonstrated the uniform imiquimod loading with no change in the physical form of the API within the chitosan film matrix. In conclusion, chitosan films containing 625 μg/cm^2 imiquimod were developed with desired rate and extent of delivery of Imiquimod for single use weekly.

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Chapter 1: Background

Introduction

Innate immunity plays a major role in the recognition of pathogen-associated molecular patterns. Through pattern-recognition receptors, foreign pathogens and tumor cells can be eliminated by direct killing as well as by indirectly activating the adaptive immune system. In the treatment of basal cell carcinoma (BCC), the drug, Imiquimod, acts as a Toll-like receptor (TLR) agonist and thereby stimulates the immune system. The overall objective of this research project was to develop a topical delivery system for imiquimod that would be safe and effective for the treatment of BCC. An In-house HPLC assay for imiquimod was developed, and thereafter, imiquimod was loaded into chitosan films, and the rate and extent of release were evaluated. This introduction provides the background of BCC and its treatment by imiquimod. In addition, the rationale for the use of a chitosan film formulation is also discussed, which includes a summary of the properties and release characteristics of chitosan. The section concludes with a statement of the problem and the general experimental approach.

Basal Cell Carcinoma (BCC)

BCC is a common malignant tumor in Caucasians and accounts for 95% of non-melanoma skin cancers in the United States with approximately 4 million cases diagnosed each year. [1] The mortality associated with BCC is relatively low due to low incidence of cancer metastasis. However due to its high incidence, it remains a significant health concern. This is especially so as it can be disfiguring with adverse psychological consequences.

Exposure to ultraviolet radiation is a significant risk factor due to the induced DNA damage that in turn causes complex interactions among genes. [1] This also explains the susceptibility of Caucasians to this disease, who, have minimal protective pigmentation in the skin. BCC occurs at various anatomical locations but most commonly is found in the head and the neck along with the trunk, which typically receive the greatest exposure to UV radiation. It is slow growing, although if left untreated, there is high potential for spread to surrounding tissues. This can lead to adverse events in the affected organs, which may be very debilitating, if the eyes or nose are involved. [2]

Treatment for BCC involves surgery or non-surgical approaches. Surgical procedures include electrodessication and curettage (ED &C), surgical excision and Mohs surgery, while non-surgical approaches include radiation and systemic or topical drug therapy. [3] Although, surgery remains the most common treatment modality, radiation and drug therapy have important roles in containing BCC.

Imiquimod (Aldara™) is an imidazoquinoline amine analog related to guanosine that acts as an immune response modifier with potent indirect antiviral activity. It is a prescription medication for the treatment of BCC, genital warts as well as actinic keratosis. [4] This and related molecules directly activate the innate immune system, resulting in cytokine release and costimulatory molecular expression followed by T-cell activation. It possesses antiviral and antitumor properties due to its ability to induce production of various cytokines that enhance both the innate and acquired arms of the immune system. [5]

Imiquimod and its use in BCC

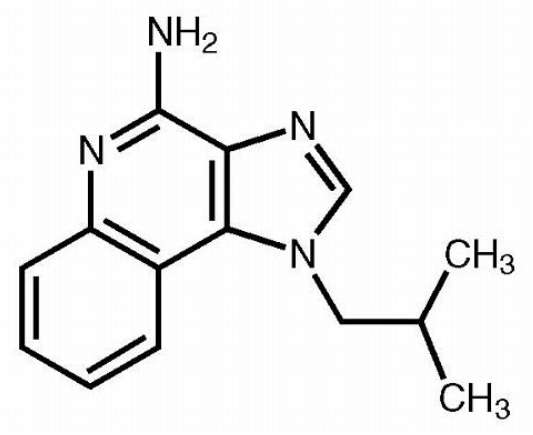

Figure 1: Chemical structure of Imiquimod

The relatively small size (M_w =240.3, pKa 5.4) and hydrophobicity coupled with a disease state located in the skin make imiquimod an ideal candidate for a topical drug delivery system. [6] Not surprisingly, good epidermal barrier penetration has been observed with topical application, e.g. the treatment of skin tumors. In several controlled clinical studies, imiquimod was shown to be effective for a variety of primary skin cancers and cutaneous metastasis of some malignancies.

The exact mechanism of action of imiquimod is yet to be fully described, but it is clear that both major divisions of the immune system are involved. Mounting a robust immune response is crucial for an effective antiviral response in diseases, such as Human papillomavirus (HPV). Imiquimod, on the other hand, does not have direct antiviral properties, but rather it induces immune reactions leading to cytokine synthesis and secretion. [5] The release of cytokines includes Interferon-α (IFN- α), interleukin (IL-6), and tumor necrosis factor (TNF-α). [7] The immune response is dependent on the

recognition of foreign antigens presented by antigen-presenting cells (APCs), such as dendritic cells (DCs), Langerhans cells (LCs), macrophages and B lymphocytes that are activated by imidazoquinoline amines. The induction of cytokines stimulates the Th-1 pathway while inhibiting the Th-2 pathway via stimulation of monocytes and DCs. This results in the production of TNF-α. [7] These pathways also activate CD8 cells that become cytotoxic T cells against tumor cells and also provide the immune memory required for future protection. [8] In addition, imiquimod also induces epidermal LCs to mature into APCs thereby facilitating the development of true T-cell mediated immunity. [9] These LCs, when activated, have increased mobility to promote migration to draining lymph nodes where they present antigen to T lymphocytes for inducing immunity. [10]

One of the major biological effects of imiquimod includes agonistic activity towards toll-like receptors (TLR) 7, and consecutively, the activation of nuclear factor-kappa B (NF-κB). The TLR family of receptors has a critical role in pathogen recognition and activation of innate immunity. [11] The innate immune system is dependent on the detection of pathogens by phagocytic cells, either through complete fixation or by binding to specific receptors, to activate the natural killer (NK) cells. Imiquimod stimulates the immune system through its TLR-7 agonist activity, thereby increasing the NK cell activity and inducing proliferation and differentiation of B lymphocytes. [12] The overall effect of Imiquimod-induced TLR-7 mediated cytokine production is a strong activation and migration of cytotoxic T cells that release perforin to destroy tumor cells. [13] Some studies have also shown that at high concentrations, Imiquimod may also exert direct pro-apoptotic activity in cultured tumor cells. [14] A schematic diagram of the biological pathways associated with the TLR agonist activity of imiquimod is depicted in Figure 2.

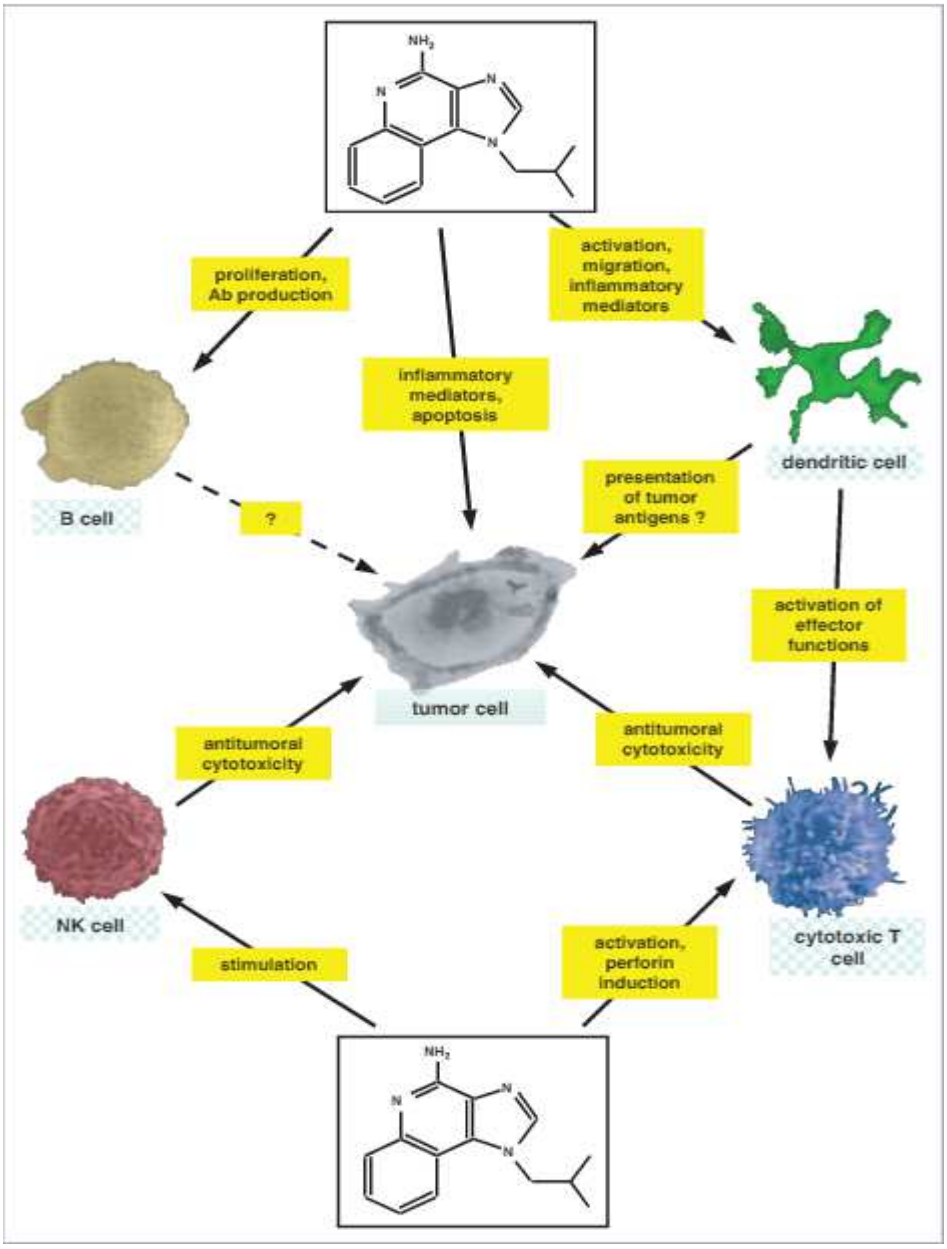

Figure 2: Schematic of the biological pathways associated with the TLR agonist activity of imiquimod (Referenced from reference 12)

Imiquimod is currently used in the case of superficial BCC or as a postoperative intervention (adjuvant therapy). Topical formulations, such as imiquimod (5% Aldara

cream), are very effective and has been approved by the FDA for treatment of superficial BCC as well as number of other diseases. [15] With topical application, it acts locally in the epidermis to enhance the Th1 response by activating the natural killer cells and stimulating B lymphocytes. [15]

Aldara™ cream is available in single 250 mg sachets that contain 12.5 mg of imiquimod.[16] The patient information leaflet dictates that the user is to apply a thin layer of cream over a treatment area of not more than about 20 cm^2. [17] It is also recommended that the cream be applied five to six times a week for six weeks and left undisturbed for 6 to 10 hours at the treatment site. [17] The cream is supplied on an outpatient basis with the intent that the patient applies the medication at home on the affected areas of the skin. Despite manufacturer's recommended dosage, the amount of cream actually applied per unit area of skin as a 250 mg sachet can be expected to depend on the patient, and studies have indicated that it may be inappropriately spread over a much larger area of 386 cm^2. [18] Hence, there is a potential for significant variability in the dose of imiquimod with self-administration that would lead to inconsistencies in the clinical response. In light of the pharmacology and the current shortcomings of the cream, it follows that a continuous delivery system, which adheres specifically to the cancerous site and provides occlusion against water and fluids, would provide a significant advantage.

Chitosan

Topical delivery possesses a distinct alternative to oral delivery as the first-pass metabolism of drug in the liver is avoided. In comparison to injectable products, there is no painful administration with needles in delivering a therapeutically effective amount of drug across the skin. [19] Perhaps of greatest significance is that there is local delivery of drug at the site of application. This provides an enormous pharmacokinetic advantage for localized skin diseases, where the concentration of the affected region of the body is maximized and the concentration to the rest of the body, and thereby systemic side effects, is minimized.

A polymer based topical patch/film would have significant advantages over the currently available cream formulation such as Aldara™. That is, the dose can be strictly controlled by the manufactured film, which will necessarily have a fixed dose that is delivered at a reproducible rate. The area of coverage can also be limited, provided the film is formulated to allow cutting to a size and shape that matches that of the affected area of the skin. In addition, these films can be manufactured with the impermeable backing membrane that provides barrier against water and body fluids and hence enhanced patient compliance.

In topical delivery systems, the polymer is the most important, non-active component. It determines the release characteristics of the drug as well as adhesion to the skin. [20] Both naturally occurring and synthetic polymers have been used for drug delivery to the skin. Natural polymers have specific properties that are not easily altered, which may pose difficulties in meeting the requirements for transdermal drug delivery. In

contrast, synthetic polymers can be synthesized with a wide range of properties but tend to have poorer biocompatibility. [19] In particular, skin irritation is common with currently available polymeric drug delivery systems.

During the past three decades, several polymeric systems for topical delivery have been explored in the field. Among the various polymer choices for film formulations, Chitosan, a natural polymer, is recognized as having many properties amenable for the delivery of imiquimod. Chitosan is naturally occurring biopolymer derived from Chitin, which is one of the most abundant, renewable natural cellulosic polymer. Chitosan is a polysaccharide composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine, that are linked together by a β-(1,4) glycosidic bond. [21] It is biocompatible, biodegradable, non-toxic and non-antigenic while possessing immunological activity. [22] Chitosan also possesses unique biological properties that include bactericidal, bioadhesion, anti-tumoral activities. There is also evidence that it promotes wound healing. [23, 24]

Due to these distinct properties, Chitosan is used in various pharmaceutical drug delivery systems. For processing, it has good solubility in organic acid solvents, allowing ready formation of films by solvent evaporation. Cast chitosan films have reasonable tensile properties, which can endure the stress exerted with physical movement in various anatomical locations in the body. [25, 26] Localized delivery of therapeutic drugs using biodegradable polymers can provide slow and controlled release over the desired period of time. Cross-linking of chitosan is an approach that has been used to control the swelling rate and thereby the release rate. [27, 28] In addition, the molecular weight of chitosan influences the drug release properties, where a high molecular weight

decreases the degradation rate of chitosan at the same degree of deacetylation. [29] In transdermal applications, Chitosan may enhance the percutaneous penetration of drug compounds by opening tight junctions in the epidermis and therefore may enhance the delivery rate of low molecular drugs. [30, 31] In this study, Chitosan was selected as the polymeric carrier of imiquimod for development of a topical drug delivery system for the treatment of BCC.

Statement of the Problem and Research Objectives

In order to address the above stated deficiencies of the cream formulations and to improve the therapeutic approach to the treatment of BCC, the aim of this project was to design, prepare and test imiquimod-loaded chitosan films. For this aim, the physicochemical characteristics of the film were characterized to elucidate those aspects required in achieving controlled delivery of imiquimod. In consideration of the treatment of BCC, the imiquimod-loaded chitosan film should be of fixed dimensions, have robust content uniformity, and provide sustained release for the duration of one week. To undertake this aim, In-house HPLC analytical method was developed, which provided an easy and sensitive determination of imiquimod during physico-chemical and release studies of the chitosan films.

Chapter 2: Development of The In-House High Performance Liquid Chromatography (HPLC) Method for The Analysis of Imiquimod

Introduction

Analysis of any pharmaceutical dosage form involves the requirement of a simple, robust, easily and rapidly performed analytical method for quantifying the concentration of the active ingredient, free of interference from other excipients. The developed analytical method should also have sufficient precision, accuracy and reproducibility to meet FDA guidelines. [32] Drug delivery systems containing Imiquimod are the subject of scientific studies for its *in vitro* release and hence there is a need for a quick, easy and inexpensive analytical method for its quantification. Analytical reports involving the determination of imiquimod are relatively scarce due to its fairly recent introduction. The HPLC methods that have been successfully employed to quantify imiquimod present certain limitations, particularly when working with small volume samples of biological origin. [33] In order to overcome such challenges and easily determine the amount of imiquimod released from our film formulations, a simple and inexpensive in-house analytical HPLC method utilizing UV detection was developed.

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Experimental

Materials

Imiquimod (≥98%, HPLC) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) Acetonitrile (ACN; Fisher Scientific, Hampton, New Hampshire, USA), Methanol (MeOH; Fisher Scientific, Hampton, New Hampshire, USA), and acetic acid (Sigma-Aldrich, St. Louis, Missouri, USA) were of HPLC grade. The water used in all experiments was purified using a Millipore filtration unit (Millipore, Bedford, USA). The HPLC used for quantifying imiquimod was a Shimadzu system that consisted of a LC-10AD pump and SPD-10A/10AV UV-vis detector and an autoinjector. A 100 mm Accucore C18 column with a mean particle size of 2.1 µm was used.

Methods

HPLC analysis of Imiquimod

The mobile phase composition was a 78:22 (v/v) mixture of 10 mM ammonium acetate, adjusted to a pH of 4.0 with acetic acid, and acetonitrile. Ammonium acetate was filtered through a 0.22 µm nitrocellulose membrane filter and degassed for 5 minutes using a bath ultrasonicator. The mobile phase was pumped through the column at a flow rate of 0.3 mL/min with a column temperature of 40°C. Prior to the first injection, the column was equilibrated for 45 minutes with mobile phase. The injection volume was 3 µL, and the total run time was set to 6 minutes. The eluent was monitored at a detection wavelength of 319 nm.

Preparation of standards

A stock solution of 1 mg/mL imiquimod was prepared by dissolving an appropriate amount of drug in a 1% acetic acid solution. This solution was then further diluted with

the acetic acid solution to prepare standard solutions ranging from 2 to 200 µg/mL. These solutions were then each diluted (1:10) in 10 mM Ammonium Acetate buffer at pH 4.0 to produce calibration standards ranging from 0.1 to 20 µg/mL. The stock solutions were kept in sealed, amber glass vials and stored in a refrigerator at 4°C.

Specificity and linearity

The specificity of the HPLC method was determined by comparing the HPLC trace to that obtained with a blank (mobile phase) at the retention time of the imiquimod peak. Identification of imiquimod peak in the standard solution was confirmed by running different concentrations of imiquimod and recording the retention time. Linearity of the method was evaluated using seven different concentrations. A 3 µL aliquot of each solution was injected in duplicate.

Accuracy

The accuracy of the method was determined by calculating the recovery in four samples containing different imiquimod concentrations in replicates of six. The mean, standard deviation and coefficient of variance (C_v) were calculated for each concentration.

Instrumental precision

The instrumental precision was evaluated by injecting six replicates of a standard solution containing 8 µg/mL imiquimod, and the relative standard deviation (RSD), retention time and area response of imiquimod was calculated.

Method reproducibility

The reproducibility of the proposed method was evaluated by injecting duplicate aliquots of samples that had been stored 4 days at 4°C and comparing the results obtained from linear regression to those obtained with freshly prepared standards.

Results

In Figure 2.1, an overlay of the HPLC chromatogram following an injection of a blank and sample containing imiquimod is given. It can be seen that there is no interference from blank sample at the retention time of imiquimod peak. This confirms the specificity of the method in the detection of imiquimod.

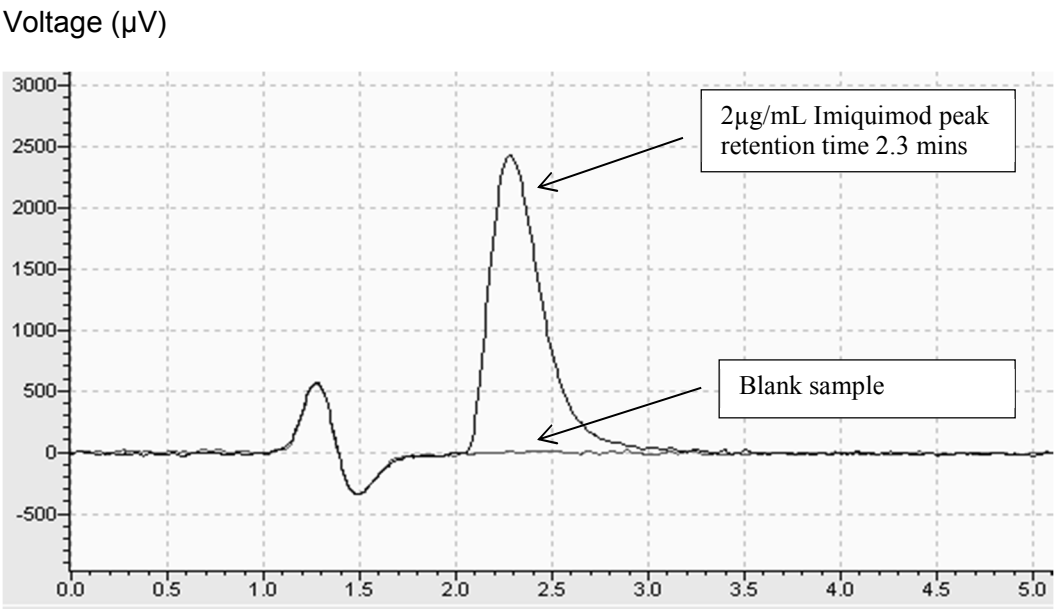

Figure 2.1: Overlay chromatogram of Imiquimod and blank sample

A calibration curve was obtained by plotting the response area from the chromatogram as a function of imiquimod concentration. The correlation coefficient from the graph was 0.9996. The standard curve of imiquimod is as shown in Figure 2.2.

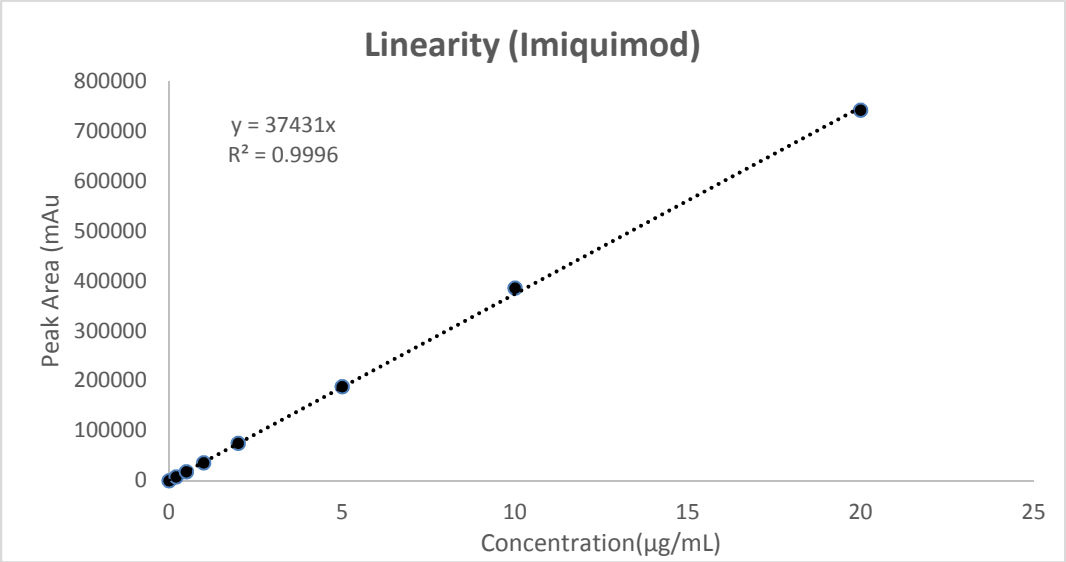

Figure 2.2: Linearity curve of Imiquimod

The accuracy of the method was calculated with three different imiquimod concentrations (0.3, 4, and 8 µg/mL). The results are as given in Table 1, where at a concentration of 0.3 µg/mL, the accuracy was 98.3%, while at 4 µg/mL and 8 µg/mL, the accuracies were 98.5% and 97.4%, respectively. The percent relative standard deviation (%RSD) for five replicates of 0.3, 4 and 8 µg/mL was found to be 2.90%, 2.54% and 1.81%, respectively. It was also observed that the accuracy was slightly lower at higher concentrations, so a lower limit of quantification (LLOQ) of 0.1µg/mL was applied. Thus, a high accuracy and low coefficient of variation was observed using this method.

Table 1: Accuracy results of Imiquimod

0.3 µg/mL Imiquimod			4 µg/mL Imiquimod			8 µg/mL Imiquimod		
Recovered Amount (µg/mL)	***% Accuracy***	***% RSD***	***Recovered Amount (µg/mL)***	***% Accuracy***	***% RSD***	***Recovered Amount (µg/mL)***	***% Accuracy***	***% RSD***
0.31	103.33	**2.90**	4.05	101.25	**2.54**	7.75	96.88	**1.81**
0.3	100.00		3.95	98.75		7.77	97.13	
0.31	103.33		3.78	94.50		7.75	96.88	
0.29	96.67		3.93	98.25		7.94	99.25	
0.31	103.33		3.98	99.50		7.93	99.13	

The instrumental precision was determined by calculating the percent relative standard deviation for six replicates at a 0.6 µg/mL standard solution and was found to be 2.18% and 0.33% for area response and retention time, respectively.

Discussion

The development of this method evolved through the optimization of chromatographic parameters that involved testing several different mobile phase compositions. As an initial start point, Ammonium Acetate buffer (pH 4.0, 10mM): ACN 85:15 (v/v) mobile phase was used. This led to a run time of 12 minutes and resulted in peak broadening. The organic phase was increased to 20% (Ammonium Acetate:ACN ; 80:20) and that led to a shorter run time of 8 minutes, however some peak tailing was observed. In order to obtain good peak symmetry and fast elution of imiquimod, an acidic pH of the mobile phase buffer was essential. The mobile phase composition that gave the best peak shape and optimal retention time was Ammonium Acetate buffer (pH 4.0, 10mM): ACN 78:22 (v/v). Using this mobile phase, the imiquimod peak had a very short retention time

of 2.3 $\pm$ 0.1 minutes that was well displaced from the solvent front. The analysis of imiquimod was carried out at a detection wavelength of 319 nm, and the run time was set at 6 minutes. This method was successfully applied in the accurate determination of drug from our film samples due to the simplicity of the technique and the short analysis time.

Chapter 3: Development and characterization of Imiquimod films

Introduction

Topical delivery of imiquimod in the treatment of BCC is a very efficient non-surgical procedure with maximum restoration of affected skin. As discussed in chapter 1, chitosan has recognized applications in tissue engineering, wound dressing and drug delivery and thus is an excellent candidate for the polymer in a polymeric film system. For this application, an analysis of the drug delivery of the currently available cream provides useful information to guide the development of film. In essence, the rate and extent of drug delivery to the skin from the film should be equivalent to that provided by the cream, taking into account the multiple dosing of the cream compared with the once-a-week application of the film.

Aldara™ cream contains 5% imiquimod in single 250 mg sachets, which is equivalent 12.5 mg of imiquimod. [16,17] Studies have shown that only about 11.5% or 1.44 mg imiquimod is released. [34] The cream is applied as a thin layer over an area not more than about 20 cm^2, which translates to a mass delivery of 0.072 mg/cm^2. Finally, the cream is applied five to six times per week or equivalently, the cream is applied every 26 to 33 hrs. [17] Taking the midpoint as 30 hrs, the flux of drug from the cream is 0.0012 $mg/cm^2/hr$. This value represents the target transport rate that provides the necessary guidance in evaluating the release characteristics of the film. As the desired duration is one week or 168 hr, the minimum total mass required in the film would be 0.2 mg/cm^2 or 200 $\mu g/cm^2$.

In the following, films were formulated with imiquimod, and the release rates were characterized. Specific parameters were examined, which included the release media, cross-linker concentration, molecular weight of the chitosan, and imiquimod concentration in the films.

Acknowledgement

The author would like to express sincere gratitude and acknowledge Dr. Buddhadev Layek for performing all the studies included in this chapter.

Experimental

Materials

Chitosan, of two molecular weights, were obtained from SigmaAldrich (St. Louis, MO, USA). Medium molecular weight chitosan (MMWC) has a molecular weight of 190-310 kDa, deacetylation degree of 75-85%, and a viscosity of 200-800 cPs. Practical grade chitosan (PGC) has a molecular weight of 190-375 kDa, deacetylation degree ≥75%, and a viscosity >200 cPs. Dulbecco's phosphate buffered saline (DPBS), and Dulbecco’s Modified Eagle Medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Propylene Glycol was purchased from Sigma-Aldrich Corp. (St. Louis, Missouri, USA) and Captisol was purchased from Cydex Pharmaceuticals (Lawrence, Kansas, USA)

Methods

Solubility determination of imiquimod

The solubility was determined by placing about 1 mg, weighed accurately, of imiquimod in a glass vial along with 1 mL of DPBS or DMEM containing a range of concentrations of captisol up to 10%. The vials were placed on an oscillating mixer set at 400 rpm held at room temperature. After 24 hrs, the vials were centrifuged at 10,000 rpm for 10 minutes, and about 800 μL aliquot was drawn through a 0.45 μm syringe filter. The filtrate was then analyzed for imiquimod concentrations by injecting 3 μL on the HPLC. For this experiment, a single sample was taken at each time point.

Preparation and characterization of imiquimod-loaded chitosan films

For film preparation, a 40 mL solution was prepared containing 1.5% (w/v) medium molecular weight chitosan, 0.5% (v/v) acetic acid and 12.31 mg of Imiquimod. With this solution, 36mL was added to an 85 mm glass culture disc and then dried for 24 hours at 40°C in a convection oven. Separate studies were conducted with different amount of chitosan and inclusion of 5% (v/v) Propylene Glycol.

The film thickness was measured by using a Marathon electronic digital micrometer (Marathon, Hong Kong) at five selected regions in the ¼" sections of four different cast films. The caliper was adjusted to the point when resistance to turning was encountered with the film. The crystallinity of the film was assessed by X-ray diffraction.

Content uniformity of Imiquimod in the films

To determine the uniformity of imiquimod in the film, six different Imiquimod film sections of 0.6 cm were cut out and were incubated with 1% (v/v) acetic acid solution for two hours followed by centrifugation. The resulting solution was filtered and analyzed for Imiquimod content using HPLC.

In vitro release measurement

The amount of Imiquimod released as a function of time was determined as follows. From the cast films, circular discs were punched out using a 1/4" single hole punch, which had a diameter of 0.63 cm (area was 0.3 cm^2). Each disc was placed in a 20 ml glass scintillation vial with 10 mL of medium. Two different media were used; 100 mM sodium acetate at pH 3.7 and DPBS at pH 7.4. The vials were placed into a thermostatted (32 °C) shaker and oscillated at 100 rpm. At 0.5, 1, 2, 4, 6, and 24 hrs, a 1 ml aliquot was taken and immediately replaced with 1 mL media. The sample was placed in a sealed test tube and stored in the refrigerator until assayed by HPLC. The experiment was carried out in quadruplets. From the assayed concentration and volume of the aliquot, the mass in each sample was calculated. After correcting for the "sampling with replacement," the cumulative mass released was expressed as a function of time.

Effect of chitosan grade on in vitro release of imiquimod

Films containing imiquimod were prepared using two different molecular weights (medium molecular weight and practical grade) of chitosan along with 5% PG. For each formulation, 0.6 cm circular disc (area = 0.3 cm^2), containing 43.4μg of imiquimod, was cut out and placed in a scintillation vial with 10 mL of DMEM containing 10% FBS. The release temperature was maintained constant at 32 °C. The time points were 0.5, 1, 2, 4, 6, and 24 hrs. At each time point, 1 mL of the release media was withdrawn and replaced with 1mL of fresh DMEM supplemented with 10% FBS. The collected release media was analyzed using HPLC for imiquimod concentration. The cumulative release percent of imiquimod as a function of time was plotted

Effect of Imiquimod content on release

For this purpose, imiquimod films containing 10 μM (8.56 μg/cm^2 film), 50 μM (42.79 μg/cm^2 film), and 100 μM (85.57 μg/cm^2 film) were prepared using 1.5 % (w/v) medium molecular weight chitosan with 5% PG. 0.6 cm circular discs (area = 0.3 cm^2) were cut out from each of the different films and placed in a scintillation vial with 10 mL of DMEM containing 10% FBS. The release temperature was maintained constant at 32 °C. Sink conditions were maintained for all films during the release study.

Results

The results from the solubility measurements of imiquimod in DPBS and DMEM as a function of captisol concentration are given in Figures 3.1 and 3.2. For DPBS, the

solubility in the absence of captisol was 1.5 μg/mL (Figure 3.1A). The aqueous solubility is reported to be about 2 μg/mL. [34] With the addition of captisol, the amount of imiquimod in solution increased in a linear manner reaching a concentration of 21 μg/mL at the highest captisol concentration of 10%. The best fit intercept and slope using linear regression were 1.00 μmol and 0.9 (μmol imiquimod/μmol captisol) in DPBS and DMEM, respectively.

In Figure 3.1B, the corresponding results plotted in moles are given. Captisol is a β-cyclodextrin derivative containing seven glucose units and has a cavity size of 0.7 nm. Imiquimod has a hydrophobic benzene ring that is sufficiently small to reside within this cavity. However, the best fit slope of the graph was only 0.0017 indicating that almost 600 captisol molecules were needed to solubilize each imiquimod molecule. Thus, while association may involve insertion of the ring into the cavity, it is apparent that the attractive energy is weak. As such, captisol is a poor solubilizer of imiquimod.

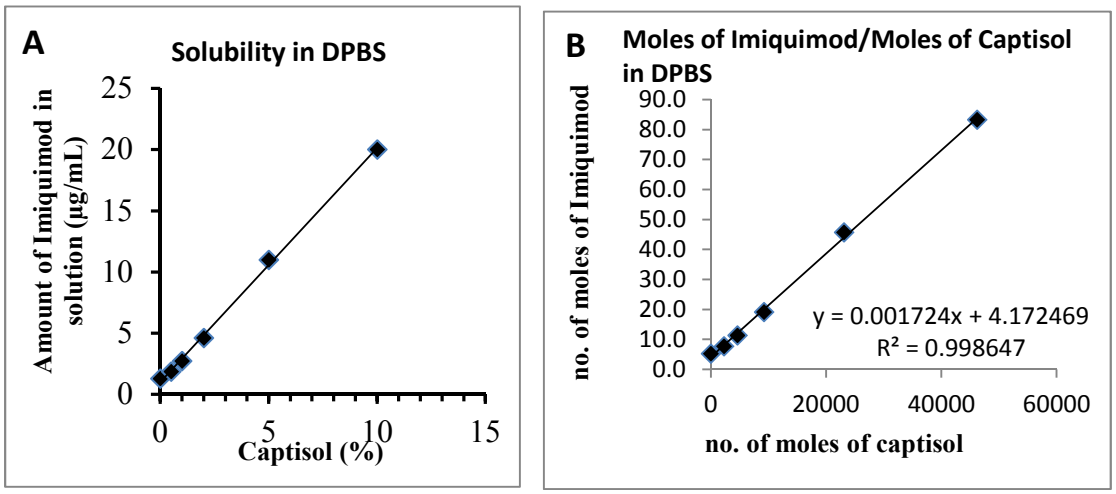

Figure 3.1: Imiquimod solubility (A) Amount of imiquimod in Dulbecco's phosphate buffered saline (DPBS) as a function of captisol concentration on a mass basis, (B) Moles of imiquimod in solution as a function of moles of captisol

In the presence of DMEM media, the solubility of imiquimod was about 9.5 μg/mL. As with DPBS, the addition of captisol increased the amount of imiquimod in solution. Specifically, the observed concentration was 25 μg/mL in presence of 5% captisol and 30 μg/mL with 10% captisol in DMEM (Figure 3.2A). However, in contrast to DPBS, the amount of imiquimod in solution was more variable and may have not increased linearly with the captisol concentration.

Discounting the possibility of nonlinearity and applying linear regression, the resulting best-fit slope with DMEM was 0.0017 mol/mol. This value is equivalent to that observed with captisol in DPBS. This indicates the solubilizing effect of captisol is indistinguishable in the two different buffer solutions, where comparable moles solubilized per mole of captisol were found.

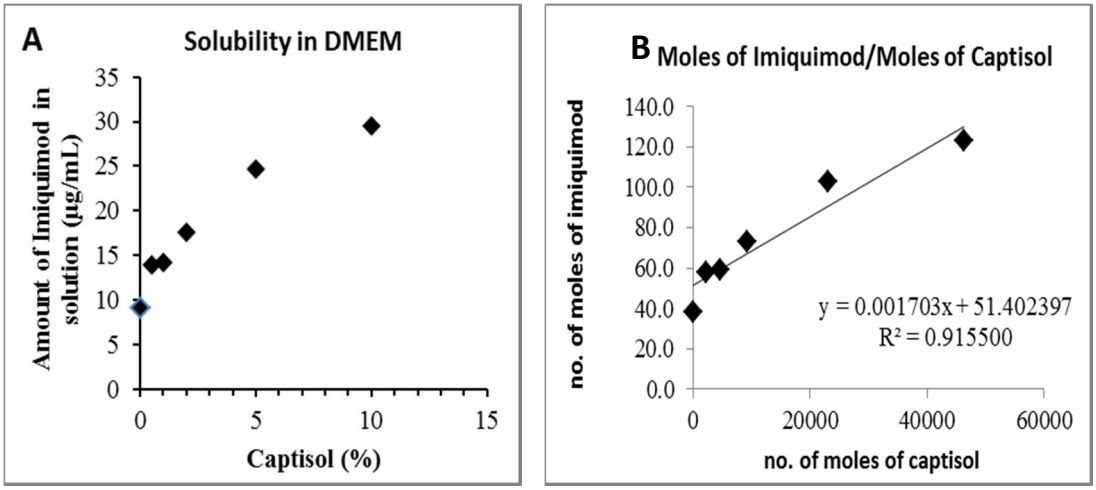

Figure 3.2: Imiquimod solubility (A) Amount of imiquimod in DMEM in presence of captisol, (B) mole ratio of imiquimod to captisol

The solubilities of imiquimod in DPBS and DMEM were 1.5 and 9.5 μg/mL, respectively. The much higher solubility observed with DMEM is likely due to the

presence of plasma proteins in the media. From pharmacokinetic studies reported in the product literature, Imiquimod is about 90-95 % bound to proteins. [34] This binding would increase the total amount of drug in solution. With the assumption that the solubility of free imiquimod remains unchanged in the presence of proteins, then 1.5 µg/mL of imiquimod is free, and the remaining 8.0 µg/mL is bound or about 84%. This is in reasonable agreement with the literature value reported above. Moreover, if the extrapolated value of DMEM from linear regression is used, the solubility value obtained would be 13.8 µg/mL and therefore the percent bound would be about 90%.

In Figure 3.3, a photograph of the Imiquimod loaded chitosan film is given. The film appears as a homogeneous, translucent circular disk.

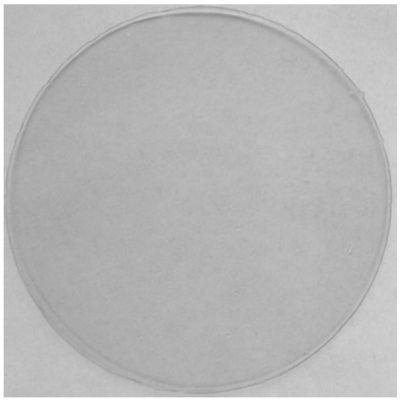

Figure 3.3: Photograph of imiquimod loaded chitosan film

In Figure 3.4, the powder x-ray patterns of Imiquimod, chitosan, and Imiquimod loaded chitosan film are given. Crystalline imiquimod has characteristic peaks with 2θ values of 11, 15, 19, 22 and 24°, consistent with the literature. [35] In contrast; chitosan has a broad peak centered near 20°. With incorporation of imiquimod into chitosan, the

characteristic peaks of imiquimod are visible and are seen to arise from a broad halo, which appears centered near 22°. These features are consistent with crystalline imiquimod being present in the film composed of an amorphous polymer. It is known that as the particle size decreases, there are fewer diffraction planes compared larger particles. With fewer planes, a reduction in peak intensity as well as broadening of the peak can be observed. It is noteworthy that the peaks arising from imiquimod, when present in the film, do not appear to be visibly broadened suggesting a relatively large particle size.

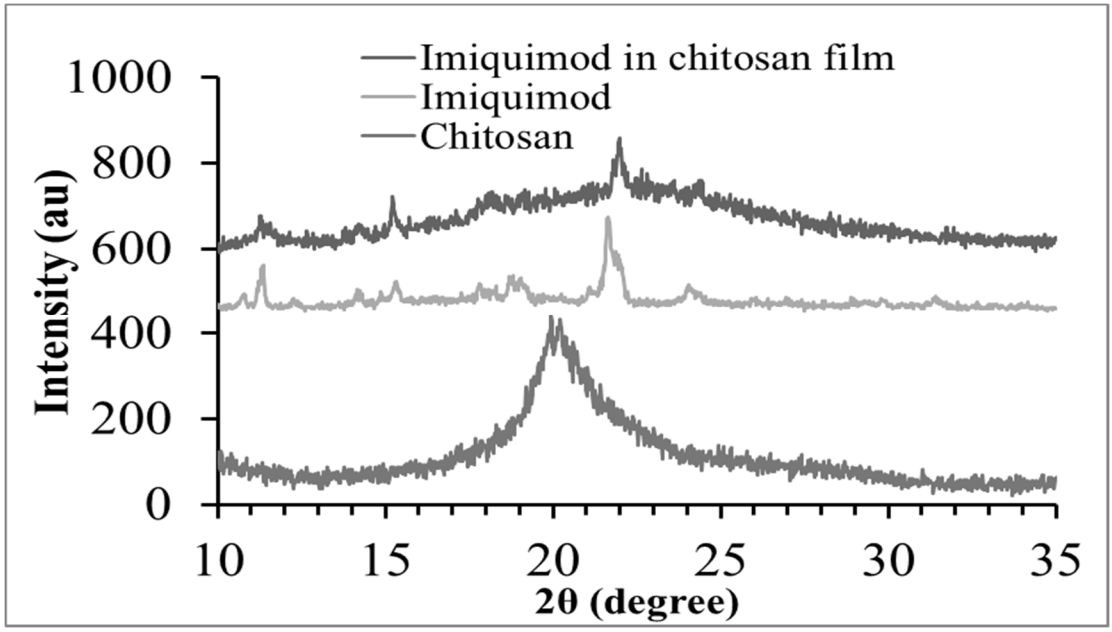

Figure 3.4: Powder x-ray diffraction patterns of Imiquimod, chitosan, and Imiquimod loaded chitosan film.

The results from measurement of the film thickness are as given in Table 3.1. The average thickness of the sections for the four different films was 0.101 mm. For the 20 measurements, the thickness ranged from 0.095 to 0.103 mm. The mean values for the four different films ranged from 0.100 to 0.102 mm, reflecting the excellent

reproducibility of the preparation method. There appears to be more variability in the thickness of a given film than in the variability between films, which is a consequence of the limitation of the measurement technique by electronic caliper. The edge of the film was thicker due to wetting of the side of the dish by the solution. However, circular punches were taken from the middle portions of the film, and the edges were avoided to yield homogeneously flat films. The mass of the 0.3 cm^2 film circle was measured to be 2.88 mg. Therefore, the density was estimated to be (2.88mg/(0.0101 cm)(0.3 cm^2) = 0.960 g/cm^3.

Table 3.1: Measured thickness at five locations (R1-R5) for four different imiquimod/chitosan films.

Thickness (mm)								
	R-1	**R-2**	**R-3**	**R-4**	**R-5**	**Average**	**SD**	**CV (%)**
Sample 1	0.103	0.105	0.095	0.102	0.103	0.102	0.004	3.79
Sample 2	0.1	0.099	0.102	0.101	0.099	0.100	0.001	1.30
Sample 3	0.103	0.101	0.1	0.101	0.1	0.101	0.001	1.21
Sample 4	0.099	0.101	0.101	0.103	0.102	0.101	0.001	1.47

The results of assessment of the content uniformity of imiquimod are as given in Table 3.2. Here, the amount of imiquimod determined in six different films is given along with the mean, standard deviation and associated coefficient of variation (CV). The average amount of imiquimod was found to be 43.36 µg with a CV of 1.76%.

Table 3.2: Amount of imiquimod assayed in six different films, mean, standard deviation and coefficient of variance.

Sample	Imiquimod amount. (µg)	Average (µg)	SD	CV (%)
Sample-1	43.11	43.37	0.77	1.77
Sample-2	44.11			
Sample-3	43.85			
Sample-4	43.66			
Sample-5	43.50			
Sample-6	41.96			

The effect of media pH on the *in vitro* release rate from imiquimod films was assessed. For this purpose, we used 1.5% (w/v) medium molecular weight chitosan (MMWC) films with imiquimod and performed the release in 100 mM sodium acetate buffer at pH 3.7 (Figure 3.5A) and also in DPBS at pH 7.4 (Figure 3.5B). From Figure 3.5A, it is seen that at a pH of 3.7, there is an initial burst release of about 80% with 100% release at the end of 2 hrs. However, at biological pH of 7.4 (figure 3.5B), a burst release of only about 2% was observed. The cumulative release increased nonlinearly, and at 100 min, the release rate was very low. At 24 hrs, only 12 % was released corresponding to 5.21 µg. Thus, much more rapid and complete release was observed at a pH of 3.7 relative to a pH of 7.4.

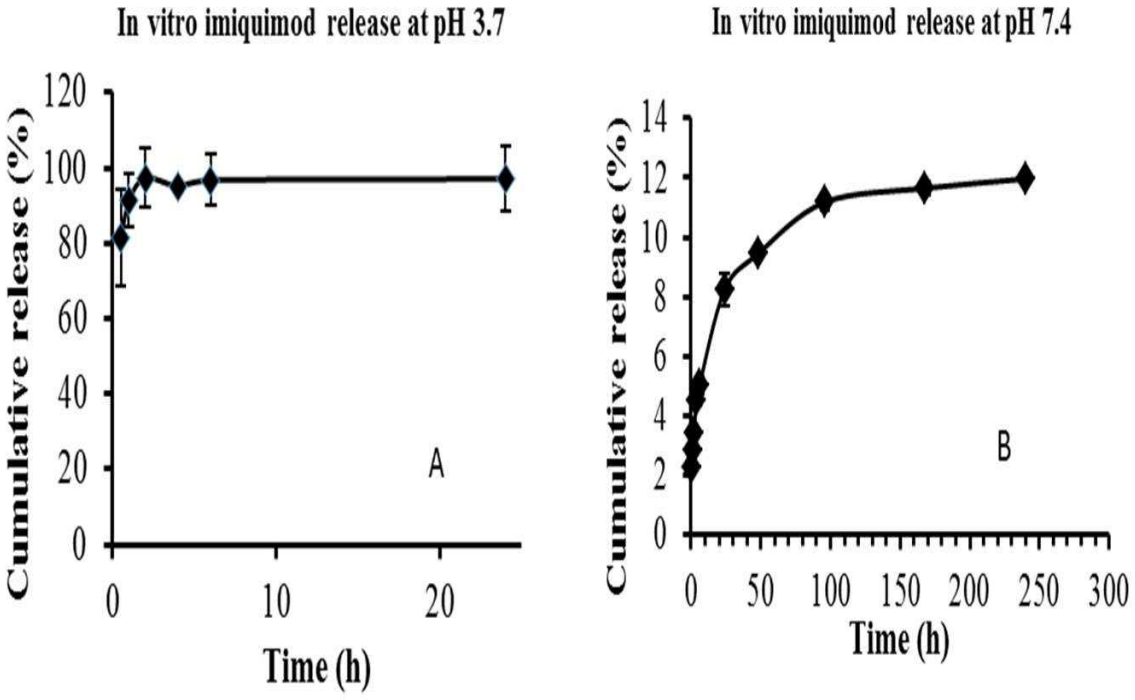

Figure 3.5: Effect of pH on *in vitro* release of imiquimod: (A) pH 3.7, & (B) pH 7.4

This phenomenon could be attributed to the pH dependence of the amount of imiquimod in solution. Imiquimod is a basic drug, and the general hypothesis is that the ionized form of a drug is infinitely soluble while the non-ionized form is soluble in a pH dependent manner. [36] Therefore, this relationship allows derivation of the following equation in determining the total amount of imiquimod in solution; where C_s is the solubility of the non-ionized form of the drug. For a weak base:

$$C_{tot} = C_s[1+ 10^{(pKa-pH)}]$$

The literature value for the pKa of Imiquimod is 5.4, the measured solubility above was 1.5 µg/mL, and the pH of the solution was 3.7. The total amount of imiquimod in solution

may be calculated as:

$$C_{tot} = C_s*[[1+ 10^{(5.4-3.7)}]$$

$$C_{tot} = 76.7\ \mu g/mL$$

Thus, with the 50-fold increase in the concentration, there would be a correspondingly large increase in the rate of drug release.

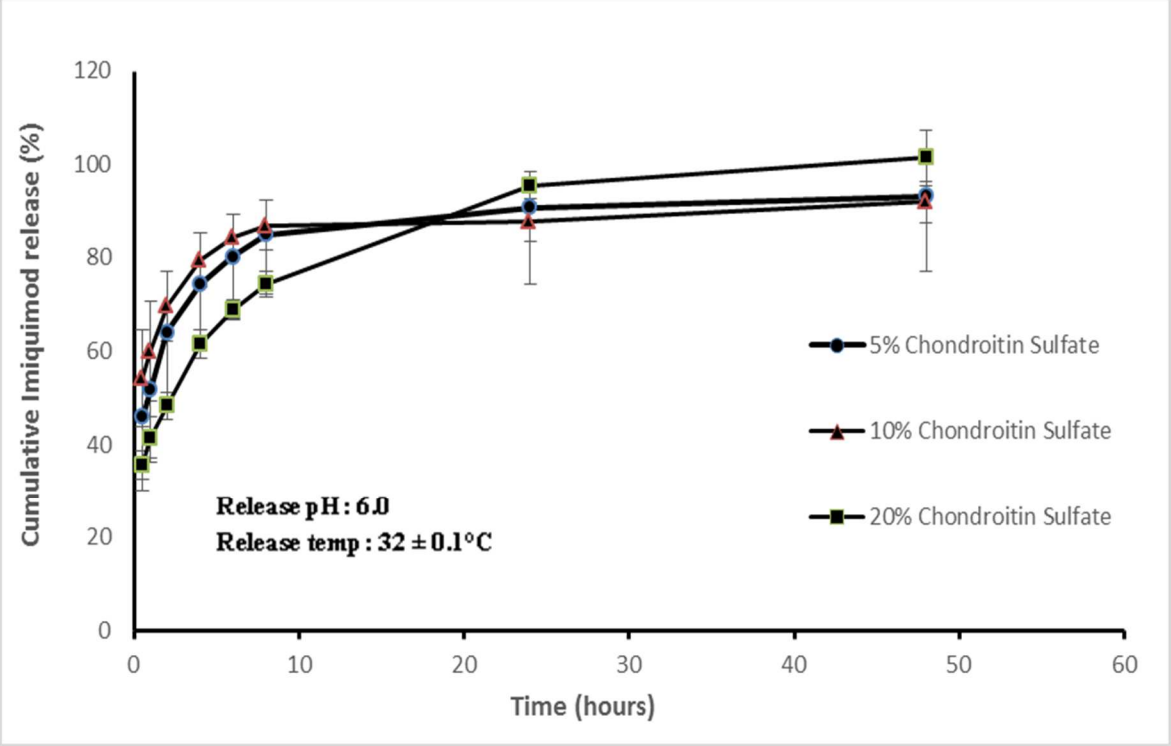

Figure 3.6: Effect of cross-linker concentration in film on imiquimod release using 5% (w/v) chondroitin sulfate, 10% (w/v) chondroitin sulfate, & 20% (w/v) chondroitin sulfate

The effect of cross-linker concentration on the release rate of imiquimod from the films was also assessed. For this purpose, we used 1.5 % (w/v) medium molecular weight chitosan (MMWC) with either 5% chondroitin sulfate, 10% chondroitin sulfate, or 20% chondroitin sulfate as the cross-linking agent. The release pH was maintained at 6.

From the graphs (Figure 3.6), it is observed that in case of the 5% chondroitin sulfate cross-linked film, there was an initial burst release of about 45%. About 85% of the loaded imiquimod was released after 10 hours, followed by a very low release rate extending to 48 hours. The 10% chondroitin sulfate cross-linked film had a slightly higher burst of about 50%, proceeding to about 85% cumulative release in 10 hours similar to the 5% cross-linked film. It was observed that the presence of chondroitin reduced the burst effect and also had a modest effect to prolong the time for complete release. The presence of higher cross-linking in the 20% chondroitin sulfate film had about half the burst effect as compared to the 10% chondroitin sulfate film, thereby depicting a more sustained release reaching completion in about 48 hours.

The swelling ratio was estimated to be about 1.5 for 0.1 mm thick films at a pH of 7.4. In contrast, there was only a 2 % increase in film thickness with addition of chondroitin sulfate. Chondroitin is a sulfated glycosaminoglycan, which can undergo complexation with the positively charged chitosan. This may be expected to cause a reduction in the swelling. However, the low pH is associated with limited swelling of chitosan and thus it would appear that addition of chondroitin did not induce a further contraction of the film, which would have been evident in a slower release profile.

The cumulative imiquimod release as a function of time from the films prepared with medium molecular weight chitosan (MMWC) and practical grade chitosan (PGC) is as given in Figure 3.7. The release pH was maintained at about 7. With the MMWC, there was a burst release of 35 % followed by a nonlinear release profile, where the rate gradually slowed. About 62% of the imiquimod was release at 150 min. For the PGC, a similar profile was observed, although the burst release was larger, 26%, and the total

release was smaller, with about 60% released at 150 hrs. These results indicate that the grade of chitosan failed to have any significant effect on the release of imiquimod. In the interest of obtaining a higher release rate, the medium molecular weight chitosan was used in the final formulation.

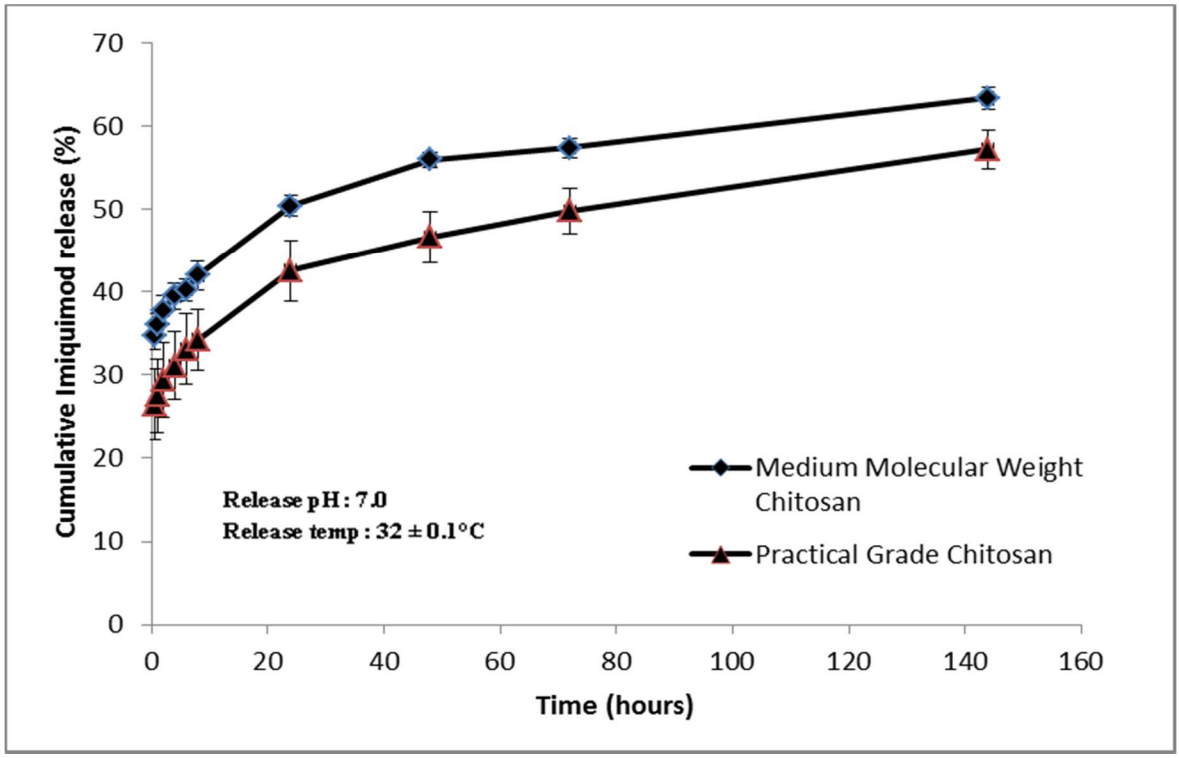

Figure 3.7. Cumulative imiquimod release as a function of time for films formulated with medium molecular weight chitosan (MMWC) and practical grade chitosan (PGC)

The results from the effect of imiquimod content in the films on the release rate are as given in Figures 3.8. In this study, three different imiquimod films containing 8.57 μg/cm^2 film (equivalent to 10 μM imiquimod in each film section), 42.79 μg/cm^2 film (equivalent to 500 μM imiquimod in each film section), and 85.57 μg/cm^2 film (equivalent to 100 μM imiquimod in each film section) were used. As seen in the graphs, the 10 μM

equivalent film section had a higher initial burst of 80%, nearing complete release in about 5 hours while both the 50 µM and 100 µM equivalent films had lower initial burst of 65-70% proceeding to about 85% cumulative release.

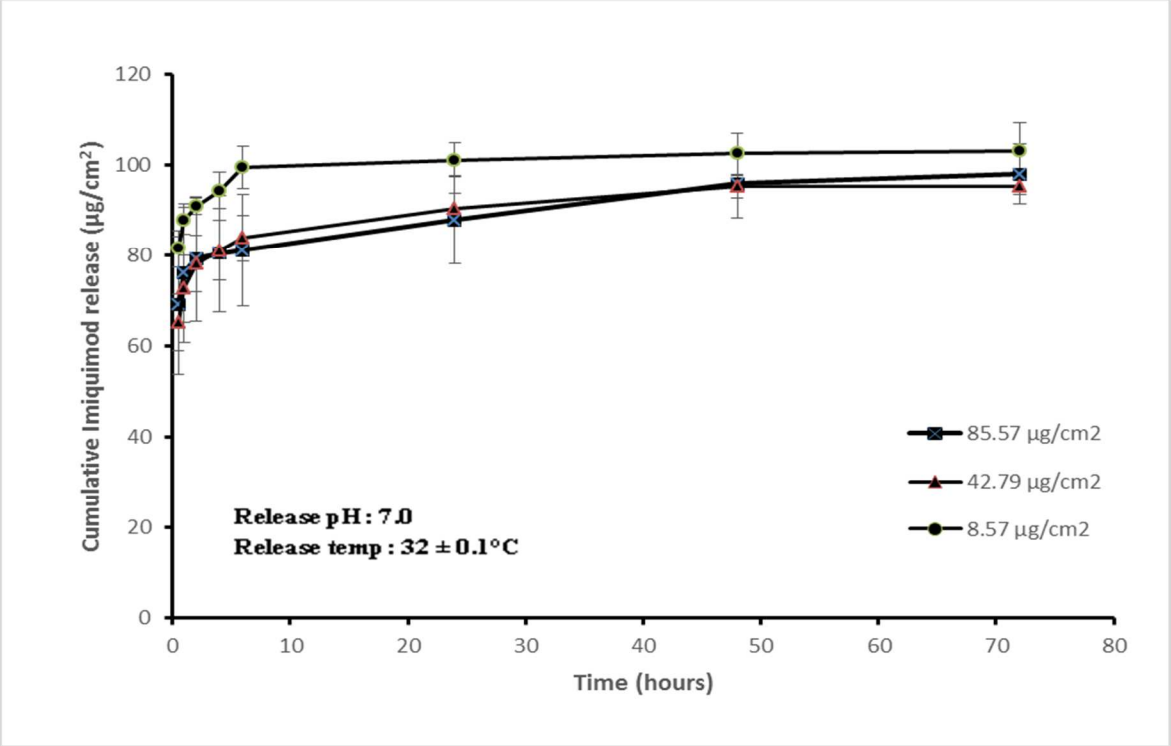

Figure 3.8: Cumulative imiquimod release as a function of time for films composed of 10µM equivalent Imiquimod per film section, 50 µM equivalent Imiquimod per film section, and 100µM equivalent Imiquimod per film section

Discussion

Conventional treatments available for skin cancers include surgery and radiation therapy, both of which are well established and widely applied. However, the potential of these approaches are limited and in some cases unsuitable for large or multiple lesions, which ultimately leads to poor cosmesis. [37] Other treatment modalities, such as curettage, and cryotherapy, are mostly effective against superficial BCC but are associated with a high rate of recurrence. [37, 38] The National Comprehensive Cancer

Network (NCCN) states that the goal of treatment for BCC is the elimination of the tumor with maximal preservation of function and physical appearance. Imiquimod has been increasingly used in the topical treatment of BCC with successful clinical outcomes while having no evidence of scarring. [39] Thus our chitosan film was formulated with imiquimod with the goal of controlled topical delivery to superficial BCC lesions. In consideration of the treatment of BCC, the imiquimod-loaded chitosan film should be of fixed dimensions, have robust content uniformity, and provide sustained release for the duration of one week.

Imiquimod-loaded chitosan films were readily formed by solvent evaporation, which resulted in an aesthetically appealing film formulation. Based on our results, the process resulted in very uniform, reproducible films with respect to thickness with a coefficient of variance (C_v) of less than 1.5%. The content uniformity was also very good in which 153.32 μg/cm^2 imiquimod was obtained. The findings from these characterization studies strongly suggested that our film composition contained a homogeneous dispersion of imiquimod into the hydrophilic chitosan, resulting in uniform thickness and content distribution across the film, which fulfills two of the criteria set for the film. In addition, the crystallinity of imiquimod inside the amorphous polymer was intact as characteristic diffraction peaks are seen in the powder X-ray diffraction pattern in Figure 3.4. This tends to favor chemical and physical stability of the formulation.

The more challenging aspects of the project relate to modulating the properties of the film to delivery drug at the needed rate and duration for a once a week application. The solubility study was necessary to identify appropriate conditions for which the release measurements could be made under sink conditions. We were able to

demonstrate that the use of sink conditions enabled a complete release within 6 hours for the films containing the smallest amount of imiquimod. Our studies also indicated the importance of pH in the release rate, which perhaps could provide a means through which the rate of release can be modulated.

Chitosan is a frequently used polymer due to its biocompatibility, biodegradability and non-toxicity. [22] Localized delivery of therapeutic drugs using chitosan are capable of slow and controlled release over the desired period of time and hence chitosan served as a desirable polymer in our film formulation for imiquimod delivery to BCC lesions. The release of biological agents from chitosan film is dependent on its swelling behavior, which is a function of the pH and is also controlled by the cross-linker. Literature indicates that the degree of swelling is inversely proportional to the degree of cross-linking. [25] The results from our release study revealed that the presence of crosslinking significantly reduced the rate compared to films without crosslinking, but no significant differences were evident when the cross-linker concentration was varied from 5 to 20% (Figure 3.6). Based on this observation along with the fact that the films would likely remain intact, when adhered to the skin, no cross-linking agent was used in our final film formulation so as to reduce one excipient from the film. The release profile from all the three cross-linker concentration films showed a near-complete release within 48 hours when the release pH was maintained at 6. Propylene Glycol was introduced in order to mitigate the brittleness of the films in the absence of a cross-linker.

Another important consideration of chitosan-based release is the effect of chitosan molecular weight on the drug release profile, where a high molecular weight is associated with low degradation of chitosan. [23] In order to examine this condition; we

incorporated both medium molecular weight (MMW) and high molecular weight practical grade chitosan (PGC) in our formulation and monitored the release. As observed, the release profiles obtained from the different molecular weight films were almost identical with a slightly higher initial burst in the case of the medium molecular weight chitosan film. Thus, no correlation with molecular weight or grade of chitosan was found. It is likely that degradation did not occur in the films used in this study, or if present, did not affect the release rate of the relatively low molecular weight, Imiquimod.

Since, further studies would require in vitro evaluation of films on supporting the growth of cells where a neutral pH of 7 would be desired. The release pH was kept at 7 for our final film formulation since our release media (DMEM substituted with 10% fetal bovine serum) had the same pH and as a result the profile obtained had a longer release span as compared to the previous release studies of different cross-linked films. The different cross-linker concentration film release was performed at a pH of 6, which falls into the pH range of skin. However, the profile obtained from the films depicted a faster rate of release as compared to our final formulation due to chitosan's higher solubility in lower pH. Thus, achieving a steady release over the course of six days, our final film formulation contained medium molecular weight chitosan as the polymer. This may prove to be helpful should the film reach manufacturing, because specifications on molecular weight need not be narrow.

Our bioadhesive, topical chitosan films were capable of releasing a defined dose of imiquimod over a period of six days. It should be noted that the maintenance of the cream in some anatomical locations of the human body would present difficulty due to factors such as moist environments and high shear forces. Previous studies have shown

that the Aldara 5% cream 250mg single sachet is capable of only releasing 11.5% of its imiquimod content. [40] The cream is applied five to six times per week, and as calculated before, the minimum total mass of imiquimod required in the film to obtain the required dosing would be 200 μg/cm^2. Our films had an imiquimod content of 153 μg/cm^2 with about 62% release over the course of six days. While apparently low, such a film would represent a good starting point in clinical testing.

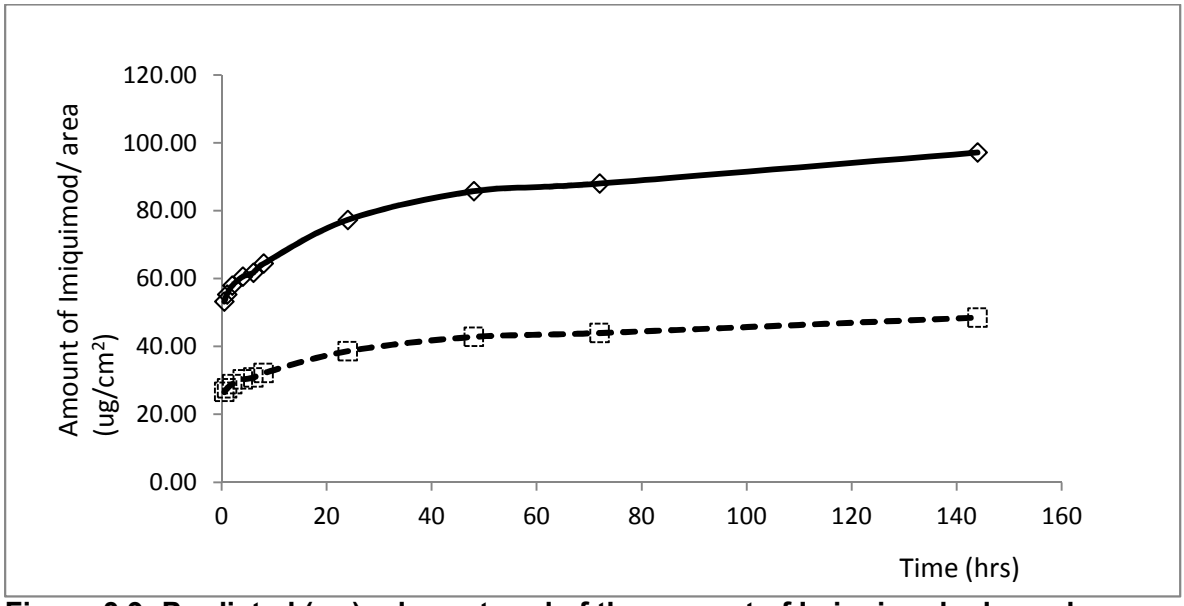

Figure 3.9: Predicted (----) release trend of the amount of Imiquimod released per unit area against time from commercial films

Figure 3.9 depicts the release profile from our final film formulation that is composed of 1.5% (w/w) medium molecular weight chitosan, where the release was measured in media composed of DMEM substituted with 10% fetal bovine serum. The solid graph shows the amount of imiquimod released per unit area from both sides of our film, as would occur in the release studies. Since, our proposed film would release its payload from only one side of the film, the dashed line represents the predicted release trend from one-side. For this estimate, we assumed that the rate of release would be

decreased by a factor of two. Hence, in this case, about 32% imiquimod release is expected within the course of one week. Therefore, in order to achieve a similar release as compared to the Aldara™ cream, the required therapeutic dosing in the film should be about 625 μg/cm^2 in order to obtain the desired flux of drug of 0.0012 mg/cm^2/hr over the course of 168 hours.

Our experimental findings also indicated that the variation of imiquimod loadings in the films across a range of 50 μM - 100 μM equivalent Imiquimod did not significantly affect the release pattern (Figure 3.8). Thus, it seems likely that an imiquimod loaded film could be formulated as an external bioadhesive bandage and could be applied on the skin lesions for a week in order for a complete, therapeutic payload release. Overall, the films manufactured contain a defined drug loading per unit area and hence could be applied in accordance with the area of the lesion in order to achieve better clinical outcomes while mitigating administration variability. This could then be used in a clinical trial involving different skin neoplasia and therefore aid in the determination of an appropriate dose of imiquimod for successful treatment outcomes. [41] Therefore, the local controlled delivery of imiquimod from a mucoadhesive film deserves further attention as an effective non-invasive treatment modality. [42]

In summary, topical delivery of drugs continues to represent an attractive alternative to other delivery routes while offering advantages such as low systemic toxicity and localized delivery. [19] Future work in topical imiquimod delivery to BCC would involve further optimization of film formulation parameters, thus enabling more control of the drug release. In order to increase the bioavailability of the poorly soluble imiquimod, the

development of a system that could form films through spray drying would be of significant interest.

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